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HIV Cell-to-Cell Transmission Requires the Production of Infectious Virus Particles and Does Not Proceed through Env-Mediated Fusion Pores

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Direct cell-to-cell transmission of human immunodeficiency virus (HIV) is a more potent and efficient means of virus propagation than infection by cell-free virus particles. The aim of this study was to determine whether cell-to-cell transmission requires the assembly of enveloped virus particles or whether nucleic acids with replication potential could translocate directly from donor to target cells through envelope glycoprotein (Env)-induced fusion pores. To this end, we characterized the transmission properties of viruses carrying mutations in the matrix protein (MA) that affect the incorporation of Env into virus particles but do not interfere with Env-mediated cell-cell fusion. By use of cell-free virus, the infectivity of MA mutant viruses was below the detection threshold both in single-cycle and in multiple-cycle assays. Truncation of the cytoplasmic tail (CT) of Env restored the incorporation of Env into MA mutant viruses and rescued their cell-free infectivity to different extents. In cell-to-cell transmission assays, MA mutations prevented HIV transmission from donor to target cells, despite efficient Env-dependent membrane fusion. HIV transmission was blocked at the level of virus core translocation into the cytosol of target cells. As in cell-free assays, rescue of Env incorporation by truncation of the Env CT restored the virus core translocation and cell-to-cell infectivity of MA mutant viruses. These data show that HIV cell-to-cell transmission requires the assembly of enveloped virus particles. The increased efficiency of this infection route may thus be attributed to the high local concentrations of virus particles at sites of cellular contacts rather than to a qualitatively different transmission process.

Two main modes of virus propagation have been described for human immunodeficiency virus type 1 (HIV-1): infection by cell-free virions and direct cell-to-cell transmission of the virus (reviewed in reference 52). Cell-to-cell transmission has been shown to be a more rapid and efficient mechanism, which avoids several biophysical, kinetic, and immunologic barriers (9, 13, 17, 56). Productive cell-to-cell infection requires interaction between the viral envelope glycoproteins (Env) on the surface of the infected cell and HIV receptors on the surfaces of target cells, leading to the formation of virological synapses (28, 40, 52). At the cell-cell contact sites, the interaction between Env and the receptors on the target cell mediates the creation of fusion pores between the two plasma membranes, which can be visualized by electron microscopy (51). It has been proposed that virions could go through these pores without extracellular budding, potentially contributing to the high efficiency of the HIV cell-to-cell transmission process (51). Direct translocation of viral ribonucleocapsid complexes through Env-induced membrane pores has been described for variants of measles virus associated with a neurodegenerative disease (11). In the present study, we explored whether productive infection may result from the delivery of HIV nucleic acids with replication potential through Env-mediated fusion pores or whether the so called "cell-to-cell" transmission requires the production of fully assembled infectious virus particles near intercellular contact sites.

To this end, we studied the transmission properties of HIV variants carrying mutations in the matrix (MA) protein that prevented the incorporation of the envelope glycoprotein complex (Env) into cell-free virions without affecting virus particle formation and Env-mediated cell-cell fusion. The MA protein, at the N terminus of the Gag polyprotein precursor, directs its intracellular transport to the plasma membrane (32, 46, 53), which is the predominant site of virus assembly in most cell types (5, 31, 46). Mutations affecting residues of the MA protein induce a range of perturbations in the virus assembly process, including a defect in particle release (24, 62), and the redirection of assembly to the endoplasmic reticulum or other intracellular compartments, in particular multivesicular bodies or late endosomes (21, 27, 47, 48, 57).

The formation of cell-free infectious virions also requires the expression of Env at sites of virus particle assembly. Env is transported to the plasma membrane following the vesicular pathway of cellular glycoproteins (23). HIV-1 Env is first synthesized as a 160-kDa precursor, which is then cleaved to generate two proteins: the mature surface protein (gp120) and the transmembrane protein (gp41), held together by noncovalent interactions. Lentiviruses such as HIV or simian immunodeficiency virus (SIV) harbor a particularly long C-terminal cytoplasmic "tail" (CT) in their transmembrane protein, which contains sequences that regulate the intracellular trafficking of Env (3, 6–8, 45, 61). Several studies have shown evidence for an interaction (direct or indirect) between the HIV Env CT and MA, leading to Env incorporation into virus particles (reviewed in reference 41). Of interest for this report, mutations in MA, notably in its N-terminal domain, impair

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Env incorporation into virus particles (18), which can be rescued by truncation of the Env CT (4, 22, 38). CT-truncated Env molecules may be incorporated into virions by a passive and less efficient process. Virions carrying truncated Env produced by commonly used adherent cell lines, such as HeLa and 293T, are infectious in single-cycle assays; however, replication of HIV carrying truncated Env can be observed in some T-cell lines (e.g., MT4), but not in many others (e.g., H9 and Jurkat) (1, 19, 20, 43, 59).

In virus-producing cells, Env and Gag colocalize at the cell surface (27). Env expression affects the polarization of Gag assembly both in epithelial cells (36, 37) and in T lymphocytes (16). In both cases, the degree of polarization depends on the integrity of the endocytic motif Y_{712}SPL in the Env CT. Focal convergence of Gag and Env is also observed at contact sites between infected and target cells (40, 52). Interestingly, in the context of cell-cell contacts, the impact of Env expression on the recruitment of Gag at contact sites appears to be cell type dependent. In Jurkat cells, Gag accumulation at intercellular contact sites is reduced by half in the absence of Env expression or in the presence of a CT-truncated Env, while Env expression and CT truncation have a negligible effect in MT4 cells (20). Gag accumulation was also independent of Env expression in macrophages establishing contacts with either T cells or other macrophages (25).

To determine whether HIV cell-to-cell transmission requires the assembly of infectious virus particles or whether nucleic acids with replication potential may translocate through Env-mediated fusion pores, we constructed HIV proviral clones carrying mutations in MA that are known to strongly impair Env incorporation into virions. Transfection of these clones results in the production of virus particles that are not infectious by cell-free transmission. Env is nevertheless expressed at the cell surface and permits the formation of fusion pores between the cells. We thus compared the properties of these MA mutants in cell-free and cell-to-cell assays. Our results show that these mutants also have a major infectivity defect in cell-to-cell assays. This is largely due to their inability to translocate the virus cores into the target cell cytosol. In addition, truncation of the Env CT partially restored Env incorporation into cell-free virus particles and rescued the penetration of MA mutant virions into target cells in both cell-free and cell-to-cell assays. These results strongly suggest that cell-to-cell transmission requires the production of infectious enveloped particles. The increased efficiency of this infection route may thus be attributed to the high local concentrations of virus particles at sites of cellular contacts rather than to a qualitatively different transmission process.

MATERIALS AND METHODS

Proviral DNA constructs. The parental proviral construct used in this study is pNL4-3 in which the BsshII-SpH1 fragment (encompassing the entire MA domain) was replaced by the corresponding fragment from HXBH10 carrying either the wild-type (WT) sequence or variants with the following mutations: L8S plus S9R (MA-A), Δ(16-18) (MA-B), W36S plus A37R (MA-C), and Δ(41-43) (MA-D) (38).

The CT-truncated (DCT) Env mutant was constructed by site-directed mutagenesis, replacing the 713Y codon in the cytoplasmic domain of the *env* gene (TCA) by a premature termination codon (TAA), using the following oligonucleotides: forward, 5′-GAGTTAGGCAGGGATACTAGTCATTATCGTTTCAGAC-3′; reverse, 5′-GTCTGAAACGATAATGACTAGTATCCCTGCCTAACTC-3′. The different MA mutations were then introduced into this new construct, replacing the BsshII-SpH1 fragment and producing the following constructs: MA-A DCT, MA-B DCT, MA-C DCT, and MA-D DCT.

Proviral constructs based on pNL4-3, but defective for Env (DEnv) (14) or for Vpu (DVpu) (54) expression, or encoding a fusion-defective Env (F522Y construct) (58), have been described previously.

Cells and viruses. 293T, HeLa, and HeLa-derived P4C5 cells (carrying a long terminal repeat [LTR]-LacZ cassette) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), with the addition of 200 μg/ml hygromycin for P4C5 cells. The MT4R5 T-cell line and the JLTRG-R5 indicator cells, which carry an LTR-green fluorescent protein (GFP) cassette (34, 44), were grown in RPMI medium with 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Viruses were produced by transfecting HeLa cells (or 293T cells where indicated) with NL4-3-based proviral constructs by lipofection (Metafectene; Biontex) or with jetPEI (Polyplus transfection) according to the manufacturers' instructions. Viral p24 production in supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) (Innogenetics).

Env incorporation assays. Virus preparations from transfected HeLa and MT4R5 cells were used to assess Env incorporation into virions. The virus-containing cell supernatant was overlaid on a 20% sucrose cushion in a Beckman SW28 tube, and particles were pelleted by centrifugation (50,000 × *g*, 4°C) for 90 min. Viral pellets were resuspended in TNE buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.5 mM EDTA) supplemented with 1% Triton X-100 and protease inhibitors. An aliquot was removed for p24 capsid (CA) protein determination by ELISA, and resuspended pellets were frozen at −80°C for a quantitative gp120 ELISA.

The quantitative gp120 ELISA was performed in Immulon 2 plates (Dynex) as described previously (35). The human monoclonal antibody (MAb) 2G12 was used for the detection of gp120 captured on the solid phase. Dilutions of purified $gp120_{IIIB}$ (Advanced BioScience Laboratories) were used to construct a standard curve. Similar ELISAs were also carried out with a pool of HIV-1-positive human sera for the detection of gp120.

HIV particle release. HeLa cells (10^5) were transfected with different proviral constructs and were seeded in 24-well plates. Forty-eight hours posttransfection, supernatants were collected, and the adherent cells were washed with phosphate-buffered saline (PBS) and lysed with PBS containing 10% Triton. p24 in supernatants and in cell lysates was measured by ELISA (Innogenetics). The ratio of p24 in supernatants to total p24 (in supernatants plus intracellular) was then calculated.

HIV infection of P4C5 cells. P4C5 cells were plated at 4,000/well in a 96-well plate 72 h before infection. Infection was performed using serial dilutions of virus-containing supernatant (5 to 100 ng of p24) in a final volume of 100 μl/well, at 37°C for 40 h, in the presence of DEAE dextran (4 μg/ml). P4C5 cells were then washed, and viral infection was assessed by measuring β-galactosidase activity in cell lysates, using a previously described colorimetric assay (39).

Analysis of cell-to-cell HIV transmission. Donor HeLa cells were transfected with different proviral constructs and were plated in 24-well plates (10^5 cells per well). Target cells (MT4R5) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (2.5 μM; Molecular Probes) for 10 min at 37°C. HeLa cells were washed to eliminate cell-free virions 48 h posttransfection, and 5×10^5 target cells were added in a final volume of 500 μl. Target cells were collected 4 h later and were cultured separately in 24-well plates. At different time points (15 h, 24 h, and 48 h), cells were fixed in 2% paraformaldehyde (PFA), and the percentage of productively infected (Gag-positive) cells was determined by flow cytometry as described below.

Flow cytometry analysis. Newly synthesized HIV Gag protein in infected cells was measured after permeabilization with PBS containing 1% bovine serum albumin (BSA) and 0.05% saponin and intracellular staining with a phycoerythrin-conjugated anti-Gag-p24 MAb (KC57;

Coulter). The percentage of Gag-positive cells was determined by flow cytometry. The percentage of GFP-positive cells was measured after cell fixation with 2% PFA. Flow cytometry data were acquired using a FACSCalibur instrument (Becton Dickinson) with CellQuest software and were analyzed using FlowJo software (TreeStar).

Cell-cell fusion. HeLa cells (10^5) were transfected with different HIV molecular clones and were plated in 24-well plates. Twenty-four hours later, 2.5×10^5 Jurkat-derived LTR-GFP target cells (JLTRG-R5) were added for 4 h in the presence of 6 μM nevirapine. HIV Env-mediated fusion allows the translocation of Tat produced in transfected cells into target cells, where it activates the LTR-GFP reporter construct. Target cells were separated from HeLa cells and were cultured for 20 additional hours (in the presence of nevirapine) to allow sufficient GFP accumulation for fluorescence-activated cell sorter (FACS) analysis. The efficiency of cell-cell fusion was then assessed by measuring the percentage of GFP-positive JLTRG-R5 cells.

***β*Lam-Vpr assays.** We relied on the previously described Vpr–β-lactamase (βLam-Vpr) assay (60) to measure the efficiency of HIV core penetration into the cytosol of target cells, using cell-free virus preparations. Virus stocks were produced by cotransfecting 293T cells with HIV proviral clones and a plasmid encoding the Vpr gene fused to the β-lactamase gene (a kind gift from Chris Aiken). Virus preparations were concentrated by ultracentrifugation (1 h, 22,000 rpm, 4°C). MT4R5 cells were then exposed to the virus preparation for 2 h at 37°C. Cells were then washed and loaded with the CCF2-AM loading kit (Invitrogen) in the presence of 1.8 mM Probenecid (Sigma). Cells were incubated for 2 h at room temperature or overnight at 16°C and were then washed and fixed. The cleaved CCF2-AM fluorescence (excitation at 405 nm, emission at 450 nm) was measured by flow cytometry on a FacsCanto II system (Becton Dickinson).

These experimental conditions were then adapted to measure virus core translocation in the context of cell-cell contacts, as described recently (10). Briefly, HeLa cells were cotransfected with an HIV proviral clone and the Vpr–β-lactamase expressor plasmid (1 μg of each plasmid for 1 million cells). After 48 h, target MT4R5 cells were added to the transfected HeLa cultures for 2 h. MT4R5 target cells were then harvested, washed, and processed as described above for the cell-free virus assay. Target cells were gated based on their sizes to exclude doublets and syncytia.

Subcellular localization of Env and MA proteins. To determine the subcellular localizations of HIV Env and MA proteins, HeLa cells were transfected with proviral constructs and were processed for immunofluorescence as described previously (35). MA was labeled using a mouse monoclonal antibody specific for the mature protein (ARP342), obtained from the National Institute for Biological Standards and Control (NIBSC) centralized facility for AIDS reagents. Env staining was performed using the anti-Env human MAb 2G12 (Polymun Scientific). Images of representative cells were acquired with a Leica TCS4D confocal microscope equipped with argon (488-nm) and HeNe (546-nm) lasers, a 60× Plan Apo oil immersion objective, and LAS-AF software. The extent of colocalization between MA and Env was measured on at least 5 cells per sample (10 Z-stacks/cell) by calculating the Manders overlap coefficients (Huygens' colocalization analyzer software). Manders' M1 and M2 coefficients measure the portion of the pixels in each channel (here red and green) that coincides with a signal in the other channel. The use of Manders' coefficients was particularly valuable for this study, because it permitted specific analysis of the punctuated signal obtained for the mature MA protein (green), occurring at sites of virus production. The M coefficient ranges from 0 to 1; here, if all green pixels matched a red signal, the M value would be 1.

Distribution of Env and matrix proteins at the cell-cell contact sites. To express MA and Env from our proviral constructs (some of which are not infectious) in MT4R5 cells, the cells were transduced by vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped viruses, produced by cotransfecting 293T cells with the proviral construct of interest and the retroviral vector pCMVR8.74 (plasmid 22036; Addgene). Transduced MT4R5 cells were then mixed with MT4R5 target cells (prelabeled for 10 min at 37°C with CellTrace Far Red DDAO-SE [Molecular Probes]) at a 1:1 ratio and were loaded onto polylysine-coated coverslips (1×10^6 cells in 500 μl). After 90 min at 37°C, cells were fixed and analyzed as described above.

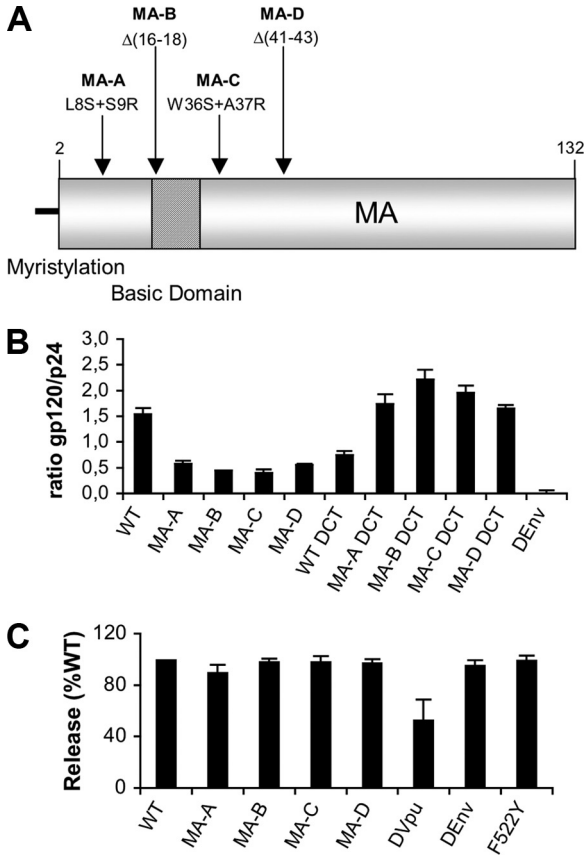

FIG 1 Characterization of HIV WT and MA mutant particles. (A) Schematic representation of the HIV MA protein, showing the positions of mutated residues. (B) Incorporation of HIV Env protein into WT and MA mutant HIV-1 particles. Virus stocks of the indicated molecular clones were obtained by transfecting HeLa cells as described in Materials and Methods. The incorporation of Env into virions was assessed by using viruses purified by centrifugation through a 20% sucrose cushion. Viral pellets were lysed in TNE buffer containing 1% Triton X-100. The concentrations of p24 and of gp120 were quantified by dedicated ELISAs, and their ratios were calculated. The results shown represent the means for two independent experiments, each conducted in duplicate wells. Errors bars indicate standard deviations. The DEnv clone does not encode an Env protein. (C) Release of WT and MA mutant HIV particles into the supernatant. HeLa cells were transfected with the indicated molecular clones, and the quantities of the HIV-1 p24 protein in the supernatant and inside the cells were determined by ELISA. The results shown represent the calculated ratio of p24 in the supernatant to total p24 as a percentage of the ratio for WT HIV. The data are expressed as means ± standard deviations for four independent experiments. DVpu is a proviral clone defective for Vpu, and the F522Y mutant encodes a fusion-defective Env protein.

RESULTS

Env incorporation into MA mutant viruses. To evaluate the requirement of Env incorporation into virus particles for cell-to-cell HIV transmission, we analyzed a panel of four HIV-1 proviral clones carrying mutations in the N-terminal one-third of the matrix protein (Fig. 1A). These mutations target sequences highly conserved among HIV-1 isolates and between HIV-1 and HIV-2

and were previously shown to abrogate cell-free virus infectivity (18). The infectivity defect was associated with dramatically impaired incorporation of viral Env glycoproteins into virions, while viral RNA incorporation and Gag precursor maturation were not affected (18). Here we first quantified the impairment of Env incorporation for the four MA mutants and the extent of its restoration by truncation of the Env CT. To this end, HeLa cells were transfected with proviral clones expressing the wild-type or mutated matrix domain and either full-length or CT-truncated Env. Virus particles released into the supernatant were purified through a sucrose cushion and lysed, and their Env and capsid (CA p24) contents were measured by ELISAs (2). As shown in Fig. 1B, the four MA mutant viruses displayed 3-fold-lower efficiency of incorporation of the full-length Env glycoprotein than the wild-type virus. The residual levels of Env incorporation were equivalent for the four mutants. The same ratio of incorporation as for wild-type MA virions was observed using both the human MAb 2G12 (Fig. 1B) and pooled patients' sera (not shown), suggesting conservation of epitope exposure; however, proper folding of the incorporated Env was not further explored.

As reported previously (1, 19, 43, 59), truncation of the Env CT resulted in a 2-fold decrease of Env incorporation into wild-type MA virions. Such suboptimal incorporation is probably due to the only passive inclusion of Env in the absence of the gp41 CT. Interestingly, incorporation of the CT-truncated Env into MA mutant viruses was highly efficient, resulting in Env/capsid ratios higher than those for HIV with wild-type MA. Increased incorporation of truncated Env into MA-mutated virions was observed previously (23, 38, 43), but its cause was not elucidated. Very similar incorporation levels were measured when virus particles were produced in a lymphoid T-cell line (MT4R5) (data not shown), thus ruling out possible cell type-specific effects.

In agreement with previous findings (18), the amounts of virus particle production in the supernatants of transfected HeLa cells, estimated here by a p24 ELISA, were similar for wild-type and MA-mutated proviral clones (not shown). Also, MA mutations did not affect the step of virus release from HeLa cells (Fig. 1C), which was measured by calculating the fraction of p24 antigen in the supernatant compared to the total p24 antigen in the tissue culture well (cell-free plus cell-associated p24).

Subcellular localization of MA and Env in transfected HeLa cells. The reduction of Env incorporation by mutations in MA was attributed to steric hindrance between MA and the Env CT, reducing their colocalization at virus assembly sites. Truncation of the CT would relieve such an obstacle (23, 38, 42). To analyze the extent of colocalization between Gag and Env at virus budding sites in transfected HeLa cells, we performed confocal microscopy analysis using an antibody that specifically binds the mature form of MA. The epitope recognized by this antibody is at the C terminus of MA, and its exposure depends on proteolytic separation from the adjacent CA domain, occurring in the late stages of virion formation. As shown in Fig. 2, colocalization of mature MA (green) and Env (red) was readily detected in HeLa cells expressing wild-type HIV proteins (yellow patches). The extent of colocalization at virus budding sites was calculated using the Manders overlap coefficient (M), by setting the region of interest in the green (MA) channel. All four mutations in MA dramatically reduced the colocalization of mature MA with full-length Env. Colocalization was largely restored by expression of a CT-truncated Env for all MA mutants, in agreement with the model described above. Thus, the reduction of Env incorporation and its rescue by truncation of the CT correlated with the extent of colocalization between Gag and Env at virus budding sites.

Infectivity of MA-mutated virions. We next analyzed the consequences of mutations in MA for cell-free virus infectivity in the presence of full-length or CT-truncated Env. Reporter cells (P4C5 cells, which are $CD4^+$ HeLa cells stably transduced with an LTR-LacZ construct) were exposed to serial dilutions of p24-normalized supernatants from transfected HeLa cells. The efficiency of infection was determined by a colorimetric assay measuring the induction of the LacZ reporter gene by newly synthesized Tat protein in target cells.

As shown in Fig. 3, and in line with previous data, virions produced by MA mutant clones encoding full-length Env did not generate detectable infection in this single-cycle assay, highlighting a defect in the early steps of virus replication. Truncation of the gp41 CT in the presence of wild-type MA resulted in relatively inefficient infection compared to that by the wild-type virus, as previously published (19, 29). This is most likely the result of the suboptimal Env incorporation measured above. For the two clones carrying mutations in the N-terminal part of MA (MA-A and MA-B), the infectivity defect was in large part due to impaired Env incorporation, since truncation of the gp41 CT, which restored Env incorporation, reduced the infectivity defect. For the other two MA mutant clones (MA-C and MA-D), CT truncation allowed only minimal infection of reporter cells, arguing for the requirement of wild-type MA residues at these positions for the efficient completion of early steps of virus replication.

Consistent with these data, exposure of MT4R5 cells (a T-cell line) to MA-mutated virions produced in the presence of full-length Env failed to generate detectable infection over a 12-day follow-up period (data not shown). Env CT truncation allowed the replication of the wild-type MA virus clone and of MA-A and MA-B viruses in MT4R5 cells, though with delayed kinetics, while propagation of the MA-C and MA-D viruses was detected in some but not all experiments (data not shown), indicating threshold levels of residual infectivity for these two mutants.

Cell-to-cell transmission of MA mutant viruses. Having determined that the infectivity defect of two MA mutants was in large part due to the inefficient incorporation of Env into virus particles released into the supernatant, we asked whether these MA mutants could propagate in culture by cell-to-cell transmission. We used a previously described approach to measure the efficiency of direct cell-to-cell HIV transmission (2, 10, 56, 58). Transfected HeLa cells were cocultured for 4 h with MT4R5 cells, after which MT4R5 cells were removed and cultured for 48 h to reveal transmission events that had taken place during the short-term coculture. Similar results were obtained when cells were cocultured for 2 or 8 h. The percentage of MT4R5 cells expressing newly synthesized Gag was measured over time by intracellular Gag staining and FACS analysis. The passive transfer of Gag (in the absence of productive infection) from transfected cells to target cells can be estimated using the F522Y fusion-defective HIV clone.

For wild-type HIV, the percentage of Gag-positive MT4R5 cells increased during the 48 h of follow-up, indicating efficient initial transmission of the virus (Fig. 4A). In contrast, the percentage of Gag-positive cells obtained for the four MA-mutated viruses did not exceed the level of the control (fusion-defective) F522Y virus, showing that mutations in MA, which prevent cell-free virus infectivity, also preclude cell-to-cell transmission of

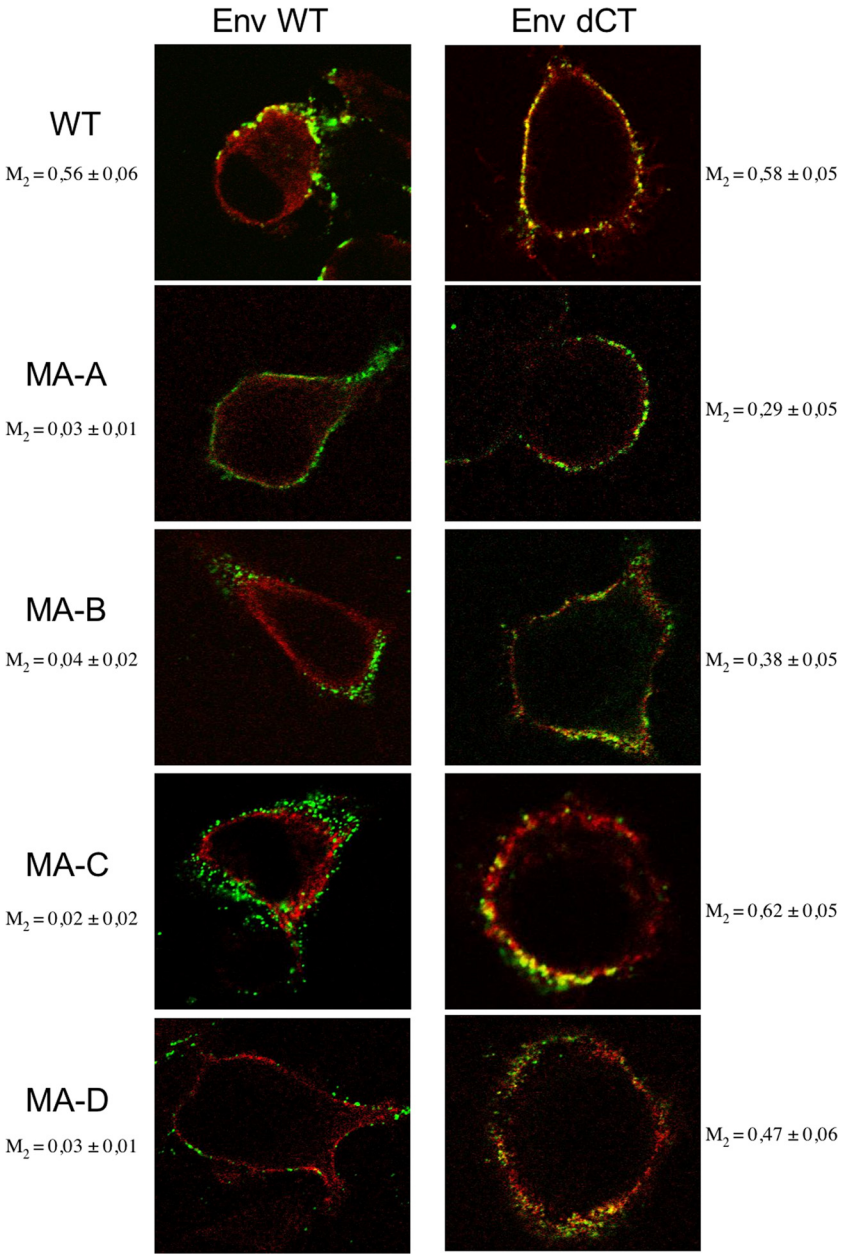

FIG 2 Analysis of the subcellular localizations of the HIV matrix protein and Env by confocal microscopy. HeLa cells transfected with the indicated molecular clones were fixed and processed for immunofluorescence analysis with anti-Env MAb 2G12 (red) and a mouse MAb specific for the mature MA protein (green). Representative confocal images for each clone are shown. The Manders overlap coefficient (M) was calculated by setting the area of interest in the green channel (MA) and analyzing 5 cells per sample (10 Z-stacks/cell).

HIV (Fig. 4A and C). We have previously shown that under these conditions the contribution of cell-free virus infectivity to intracellular Gag staining is negligible (56). In the experiment for which results are shown in Fig. 4A, cell-free virus infection, measured by the incubation of target cells for 4 h with the supernatant of HeLa cells transfected with a wild-type construct, resulted in only 2% Gag-positive cells 48 h postexposure (not shown).

In this cell-to-cell transmission assay, truncation of the gp41 CT restored the infectivity of the MA-A mutant and, to lesser extents, those of the MA-B, MA-C, and MA-D mutants (Fig. 4B and C), as in the cell-free virion assay. Finally, as for cell-free virions, transmission of wild-type MA virus with CT-truncated Env was less efficient than that of fully wild type virus (Fig. 4C). The correlation between the infectivity of cell-free virions and the competence of cell-to-cell transmission strongly suggests that these two modes of HIV propagation are not qualitatively different.

Defining the defective step in cell-to-cell transmission of MA mutants. To investigate the defective step in the cell-to-cell transmission of HIV MA mutant viruses, we first analyzed whether MA mutations perturbed the efficiency of Env-mediated cell-cell fusion. We thus established a short-term (4-h) coculture of trans-

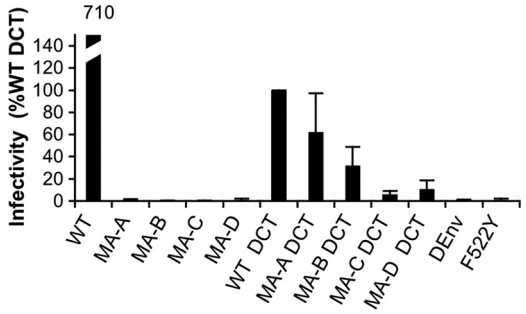

FIG 3 Single-round infectivity of WT or MA mutant HIV particles in a cell-free virus assay. P4C5 cells were exposed to a virus produced in HeLa cells. Four serial dilutions of each virus stock were tested in 96-well plates. After 48 h, infection of the target cells was assessed by staining with chlorophenolred-β-D-galactopyranoside (CPRG). The infectivity of each virus is expressed as the percentage of WT DCT infectivity. The data are expressed as means ± standard deviations for three independent experiments.

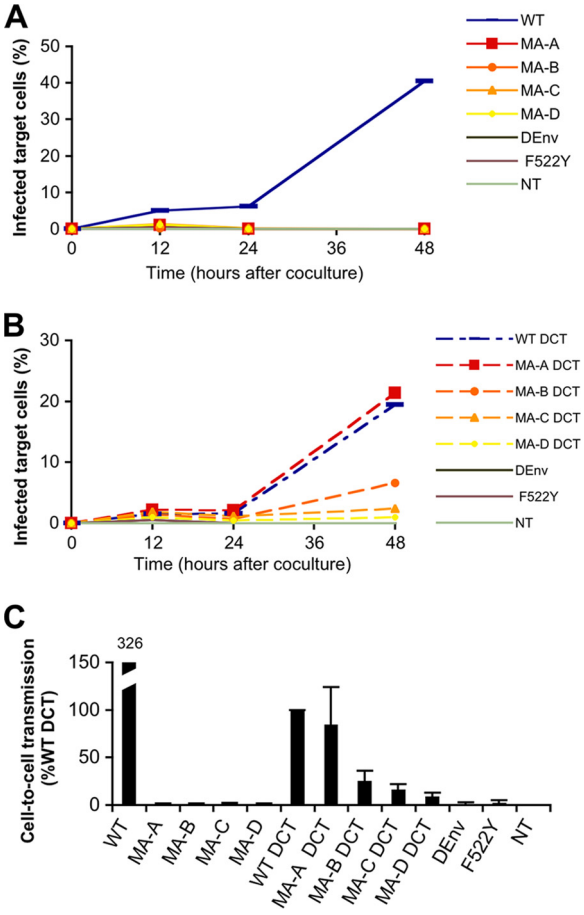

FIG 4 Measurement of cell-to-cell transmission by flow cytometry. (A and B) HeLa cells were transfected with the indicated molecular clones. Twenty-four hours later, HeLa cells were washed to eliminate free virions, and MT4R5 target cells (labeled with CFSE) were added for 4 h at 37°C. Target cells were then collected, and the percentages of Gag^+ cells were determined by flow cytometry at 15 h, 24 h, and 48 h after coculture. The results of one representative experiment are shown for clones expressing wild-type Env (A) and CT-truncated Env (B). (C) For each clone, the area under the curve was calculated and expressed as a percentage of that for the WT DCT clone. The data are expressed as means ± standard deviations for three independent experiments.

fected HeLa cells with JLTRG-R5 indicator target cells, which carry an LTR-GFP cassette (34, 44), and analyzed the levels of Tat-induced GFP activity in target cells. In this setting, Tat expressed in producer cells may translocate to target cells through Env-induced fusion pores and may induce GFP expression. To prevent the participation of *de novo*-synthesized Tat, we verified that MA mutants could not productively infect this reporter cell line by separating target cells from transfected HeLa cells and culturing them in isolation for 48 h, after which cells were stained for intracellular Gag expression (data not shown). In addition, target cells were treated with nevirapine for the entire duration of the experiment. The reference strain used in these experiments, WT(RT^-), expresses wild-type MA and Env sequences, but two mutations in the reverse transcriptase (RT) catalytic site prevented its replication. As shown in Fig. 5, we confirmed that expression of functional Env was required for this assay, since transfection of a clone carrying a deletion in *env* (DEnv) yielded negative results. Transfection of MA-mutated proviruses resulted in GFP levels ranging from 70% to 120% of those with the WT(RT^-) virus, showing that MA mutations did not have a major impact on the efficiency of cell-cell fusion.

We next analyzed the efficiency of the translocation of virus cores into target cell cytoplasm. To this end, we adapted the Vpr-βLam protocol (12, 60) to the situation in which HIV is transmitted from cell to cell (10). The Vpr-βLam assay relies on the incorporation of the fusion protein Vpr–β-lactamase into HIV virions (12, 60), mediated by the interaction of Vpr with p6, at the C terminus of the Gag precursor (33). Release of virus cores into the cytoplasm of target cells preincubated with a cytosolic β-lactamase substrate results in the enzymatic cleavage of the substrate, which can be visualized by FACS analysis. In our setting, HeLa cells were cotransfected with MA mutant proviral clones and a vector encoding a Vpr–β-lactamase fusion protein. MT4R5 target cells were then added to transfected HeLa cells for 2 h, after which they were separated and were analyzed by FACS. Figure 6A shows the results of a typical experiment in which wild-type HIV efficiently delivered Vpr-βLam to target cells. In the absence of a functional Env (DEnv or F522Y mutant), no βLam activity was detected, confirming that Env-mediated fusion is required for the delivery of Vpr-βLam to the cytosol of target cells (Fig. 6B). All four MA mutants, when expressed in the presence of full-length Env, were characterized by a 20- to 45-fold impairment of Vpr-βLam delivery (Fig. 6B). The residual β-lactamase activity observed for MA mutants may be attributed to the transient mixing of the cytosols of donor and target cells at contact sites.

Thus, MA mutations thwart the release of virus cores into the cytoplasm of target cells (Fig. 6B), although they do not affect the formation of intercellular fusion pores (Fig. 5). This phenotype strongly supports a model of HIV cell-to-cell transmission relying on the assembly of enveloped virus particles in the proximity of cell-cell contact sites, rather than the possibility of direct transmission through fusion pores. Also in this setting, the truncation of the Env CT compensated for MA mutations and resulted in virus core translocation (Fig. 6B).

In agreement with the model of cell-to-cell transmission requiring the production of enveloped virus particles, we obtained concordant results when the Vpr-βLam assay was performed with free virions (Fig. 6C). Specifically, virus particles produced by MA mutant clones expressing full-length Env tested negative in the

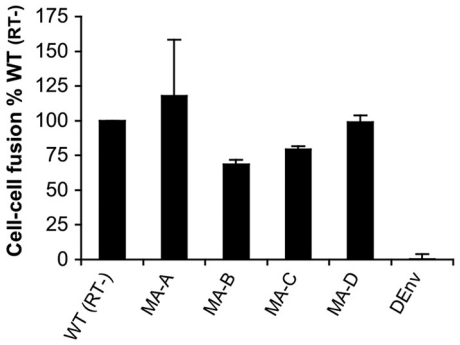

FIG 5 Fusion between transfected HeLa cells and JLTRG-R5 reporter cells. HeLa cells were transfected with the indicated molecular clones. The WT(RT$^-$) clone expresses wild-type MA and Env sequences, but its replication is prevented by 2 mutations in the RT catalytic site. Twenty-four hours later, HeLa cells were washed, and JLTRG-R5 target cells were added for 4 h in the presence of nevirapine, after which they were removed and cultured for 20 h. Cell-cell fusion efficiency was assessed by measuring Tat-induced LTR-GFP expression. All viral clones for which results are shown are replication defective, and the GFP signal is due only to Tat protein from donor cells that penetrates into target cells. Results are represented as the percentage of the result for the WT(RT$^-$) clone, based on three independent experiments.

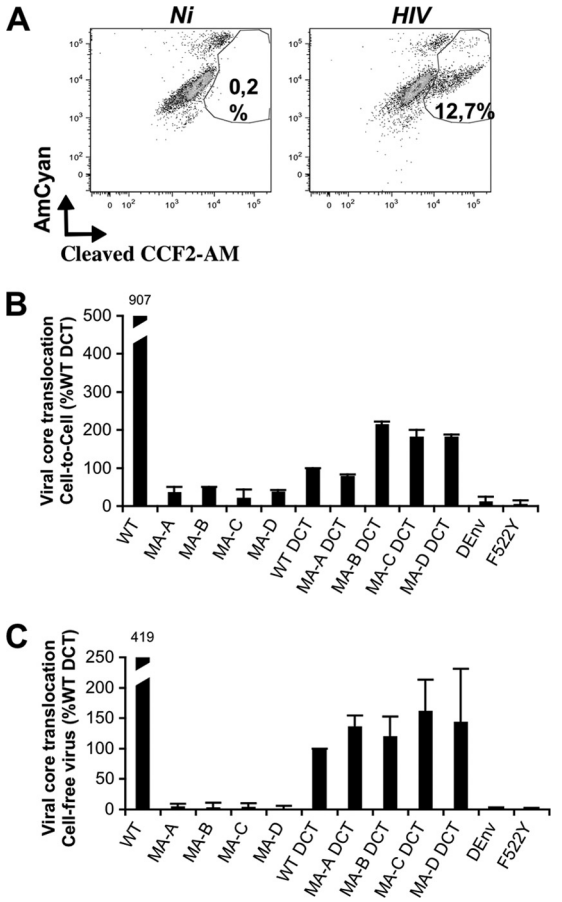

FIG 6 Translocation of the viral core into the cytoplasm of target cells for WT HIV and MA mutants. MT4R5 cells either were cocultivated with donor HeLa cells for 2 h or were directly exposed to Vpr-βLam cell-free virus for 2 h, harvested, and incubated at room temperature for 2 h with CCF2-AM. Viral fusion was evaluated by measuring the percentage of cells positive for the cleaved form of CCF2-AM. (A) Flow cytometric analysis for one representative experiment. Ni, noninfected control cultures. (B and C) Percentages of cells positive for cleaved CCF2-AM in the context of a cell-to-cell assay (B) or a cell-free virus assay (C) carried out in three independent experiments. The data are expressed as percentages of the value for WT DCT and are means ± standard deviations.

Vpr-βLam assay, while truncation of the CT of Env restored the capacity of virus particles to translocate the enzyme into target cells (Fig. 6C). Interestingly, when complemented by truncated Env, the four MA mutants displayed very similar efficiencies in this Vpr-βLam assay, confirming that competence for steps beyond virus core penetration does not affect the results of this assay.

Overall, our findings show a complete correlation between data obtained by cell-free and cell-to-cell HIV transmission, strongly suggesting that cell-to-cell transmission of HIV requires the production of infectious virus particles. The higher efficiency of this mode of transmission can thus be attributed to the high local concentration of virus particles released in the proximity of the viral synapse.

Gag and Env colocalization at the cell-cell contact sites in T lymphocytes. We have shown that mutations in MA dramatically reduced the colocalization of mature MA and full-length Env in transfected HeLa cells (Fig. 2). We next analyzed the subcellular localization of these two proteins at sites of contact between T cells. To express MA mutant clones in MT4R5 cells, these cells were transduced with a lentiviral vector (R8.74) pseudotyped by the VSV-G envelope protein and carrying the genomic RNA of our HIV MA mutant viruses. Transduced MT4R5 cells were then cocultured for 90 min with noninfected, Far Red dye-labeled MT4R5 cells. Cells were fixed, and cell-cell contact sites were then analyzed by immunofluorescence confocal microscopy, as described above. In agreement with our results for HeLa cells, MA mutant proteins displayed levels of colocalization with full-length Env markedly lower than those of WT MA protein (Fig. 7A). This defect was largely restored by Env CT truncation (Fig. 7B), confirming in a T-cell line our previous observations for epithelial cells. Interestingly, mutations in MA did not affect the accumulation of Gag at intercellular contact sites. Also, the truncation of the Env CT did not seem to perturb the formation of virological synapses. These results strongly suggest that the defect in colocalization between mature mutated MA and full-length Env and, as a consequence, the reduction of Env incorporation into assembling virions are the main mechanisms for the observed defect in cell-to-cell transmission.

DISCUSSION

To determine whether cell-to-cell transmission of HIV requires the coordinated assembly of Gag and Env to form infectious virus particles, we prevented the incorporation of Env into virions while preserving particle release and Env fusogenicity. To this end, we inserted into the N-terminal one-third of the MA protein mutations that were previously shown to have a dramatic effect on the incorporation of Env into budding particles (18). Env incorporation was restored by truncation of the Env CT, leading for some mutants to the production of infectious extracellular virus particles (38). We thus compared viral clones, carrying different combinations of MA and Env alleles, for their competence at transmission following the cell-free and cell-to-cell transmission routes. Several observations from the data described here argue for a common transmission mechanism for the cell-free and cell-to-cell

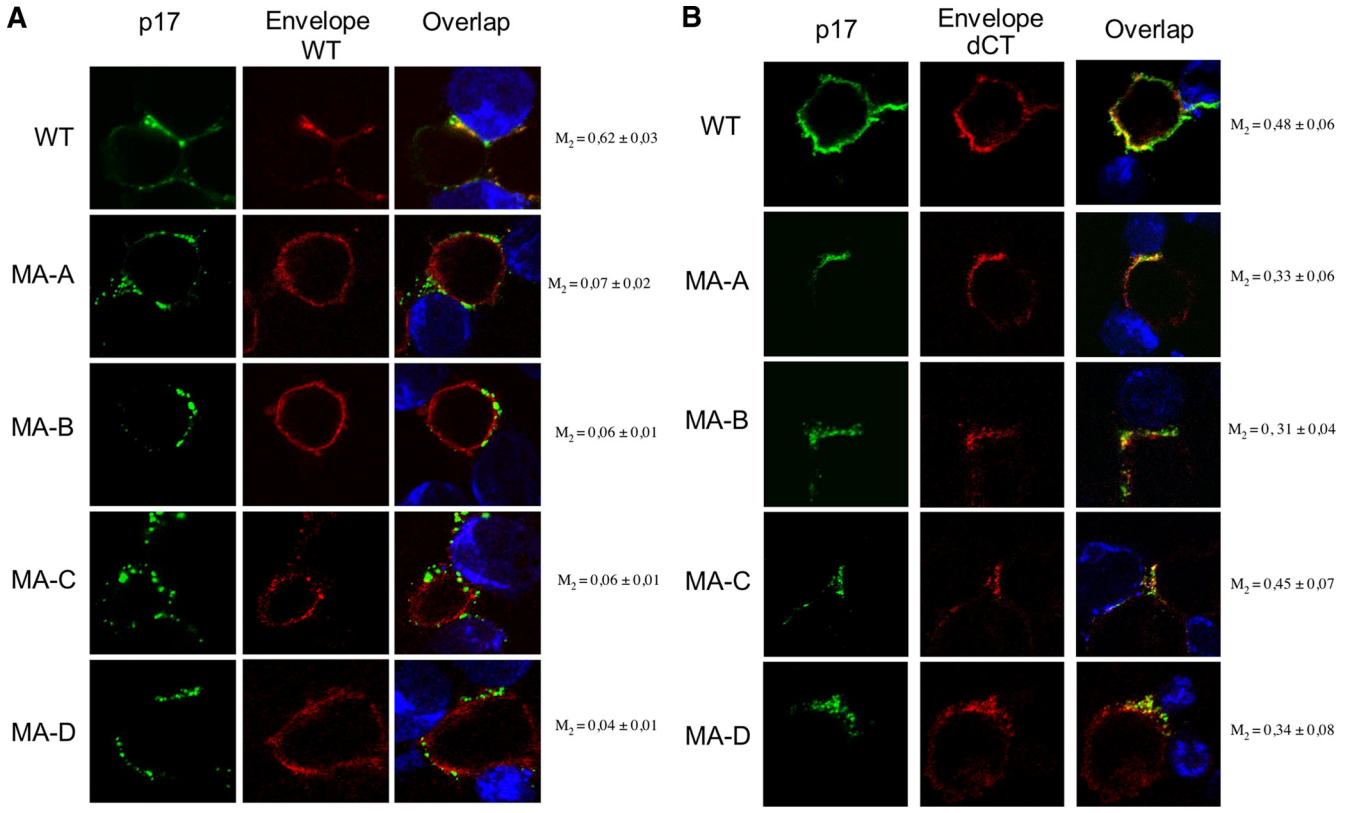

FIG 7 Localization of mature HIV MA protein and WT or truncated Env in a coculture of transduced MT4R5 cells and target MT4R5 cells. MT4R5 cells were transduced with VSV-G-pseudotyped lentiviral vectors carrying the RNAs of different MA mutant clones with WT or truncated Env genes. HIV-expressing MT4R5 donor cells were mixed in a 1:1 ratio with Far Red dye-labeled recipient cells (blue) for 1 h 30 min at 37°C. Cells were then stained for MA (green) and Env (red) and were analyzed by confocal microscopy in the context of WT Env (A) or CT-truncated Env (B). The Manders overlap coefficient (M) was calculated by setting the area of interest in the green channel (MA) and analyzing at least 5 HIV-positive cells establishing contacts with neighbor cells (10 Z-stacks/cell).

HIV transmission pathways, both of which rely on the production of infectious virus particles.

The first line of evidence supporting a common mechanism is that despite the difference in the magnitude of infection in the two sets of assays, only the combinations of MA and Env that produced infectious extracellular virions were also competent for cell-to-cell transmission. This finding strongly suggests that the formation of replication-competent cell-free virions is a common requirement for the two means of transmission and argues against qualitatively different mechanisms. In a previous study, analyzing the coevolution of MA and Env in an HIV-infected patient, we identified one combination of MA and Env alleles characterized by strongly impaired Env incorporation into particles (2). As a consequence, the single-cycle infectivity of cell-free virions was dramatically decreased (although not abolished), while virus propagation in culture proceeded through cell-to-cell transmission with a limited delay (2). In the present study, complete abrogation of cell-free virus infectivity by MA mutations prevented the potential compensation by the concentration of virus production at cell-cell contact sites and revealed the tight requirement for the assembly of infectious virions also for cell-to-cell HIV transmission.

In addition, among clones carrying truncated Env, the levels of infectivity of MA mutants, compared to that of the wild-type matrix clone, were conserved between cell-free and cell-to-cell transmission assays. MA-A displayed 15 to 30% reductions in infectivity in both experimental systems, while the residual infectivity of MA-B ranged within 30% of that of the reference strain, and those of mutants MA-C and MA-D were clearly lower (Fig. 3 and 4). The similarity of the values obtained in the two assays, despite their obvious experimental differences, suggests that the key determinants of infectivity are common. Of note, the incomplete restoration of MA mutant infectivity consequent to Env truncation shows that the targeted MA residues are also implicated in additional early steps of HIV replication. Indeed, MA mutants incorporated higher levels of truncated Env than did the wild-type MA clone (Fig. 1). Accordingly, MA mutants were at least as effective at releasing virus cores into the cytoplasm of target cells as the virus with wild-type MA (Fig. 6C). Despite this enhanced entry process, MA mutants displayed impaired completion of early steps of virus replication (Fig. 3). Previous studies have proposed a role for MA in HIV nuclear import and integration and, more recently, in viral uncoating, RNA incorporation, and cytoskeleton-mediated transport (reviewed in reference 26); some of these roles could account for the impaired replication.

Finally, the observation that MA mutations blocked HIV cell-to-cell transmission by preventing the penetration of viral cores into the cytosol also supports our conclusion that HIV cell-to-cell transmission relies on the production of infectious virus particles. Importantly, such dramatically reduced (20- to 45-fold [Fig. 6B]) virus core translocation due to MA mutations was observed despite efficient Env-dependent cell-cell fusion (Fig. 5). Thus, under

conditions allowing the exchange of cytosolic material, we did not observe productive cell-to-cell transmission. Completion of this process required truncation of the Env CT, which allows the formation of cell-free infectious virus particles. Consistently, MA-mutated cell-free virions delivered virus cores into the cytoplasm of target cells only upon truncation of the Env CT (Fig. 6B).

Given the clear phenotypes of the combinations of MA and Env studied here, the experimental approaches we used fulfilled our expectations despite a few potential limitations. It is possible that MA mutations may have perturbed the intracellular trafficking of the Gag precursor. We show, however, that the availability of a complementary (CT-truncated) Env resulted in the colocalization of Gag and Env at budding sites and at cell-cell contact sites and gave rise to particles competent for fusion with target cells. Such colocalization is likely passive, since MA-mutated Gag precursors are not supposed to establish functional interactions with the truncated Env. In contrast, in the presence of full-length Env, mutated Gag molecules could suffer from steric hindrance with the Env CT, resulting in limited access to the cell-cell contact sites where membrane fusion was taking place. Although this phenomenon may participate in reducing the efficiency of cell-to-cell transmission, it is unlikely to determine an absolute exclusion of Gag or to explain the complete loss of virus transmission for the four different mutant viruses, in view of the very dynamic and active transport processes taking place at cell-cell contact sites. Indeed, while expression of HIV Env increases the number and stability of cell-cell contacts (50), their formation and the accumulation of viral and cellular proteins also take place in the absence of Env in the cell types used here (20, 25, 50).

Another issue concerned the significant reduction in infectivity due to the truncation of Env CT in the presence of a WT MA, which limited the comparisons between viruses carrying full-length versus truncated Env. However, since MA mutations fully abrogated virus infectivity when expressed with full-length Env in both cell-free and cell-to-cell systems, our analysis did not suffer from this setback. In addition, the relatively low infectivity of DCT viruses did not prevent the establishment of a clear hierarchy for the replication competences of the different MA mutants. Finally, the use of a truncated Env to complement MA mutations limited the range of cell types to those permissive for the DCT Env variant of HIV: the MT4 T-cell line and few others (1, 43). Although the reasons for this constrained host cell range are not completely understood, a recent report strongly suggests that this may be due to the preferential spread of this variant via cell-to-cell transmission (20). Importantly, however, we confirmed that the extents of colocalization of MA and Env, as well as the efficiencies of Env incorporation into virus particles, were similar in MT4 cells and the unrelated HeLa cell line. Thus, the key parameters implicated in the processes studied here do not display a cell type specificity that would limit the value of our observations.

Taken together, the data presented here show that cell-to-cell transmission of HIV requires the assembly of enveloped virus particles. Thus, the increased efficiency of this infection route is likely to result from the high local concentrations of virus particles at sites of cellular contacts rather than from a qualitatively different transmission process. The active recruitment of HIV proteins at intercellular contacts relies on the regulated secretory pathway of infected cells (30). Virus transmission from cell to cell may overcome some conditions that are restrictive for cell-free virus infection. For instance, we have reported that this means of virus propagation limits the effect of type I interferon on HIV spread in culture (58) and that it allows the replication of viruses carrying mutations in Env that have a strong impact on the infectivity of cell-free HIV (2). In the confined space of the virological synapses, viruses are relatively protected from the environment and find elevated receptor concentrations, favoring virus entry. Accordingly, this means of virus transmission allows the simultaneous infection of cells by multiple virions (15). The penetration of multiple virus particles into the same area can facilitate infection by saturating some cellular restriction factors that would otherwise intercept incoming virus particles (49) and may reduce the effectiveness of some antiretroviral therapy (55). Further clarification of the mechanism of cell-to-cell HIV transmission may help explain some unclear aspects of HIV biology and suggest dedicated approaches to limiting virus spread.

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